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A hypothesis to explain how LaeA specifically regulates certain secondary metabolite biosynthesis gene clusters

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Abstract

Biosynthesis of mycotoxins involves transcriptional co-regulation of sets of clustered genes. We hypothesise that specific control of transcription of genes in these clusters by LaeA, a global regulator of secondary metabolite production and development in many filamentous fungi, results from its interaction with a Cys_6Zn_2 DNA-binding protein unique to the gene cluster.

Keywords: *Aspergillus flavus*, *Aspergillus parasiticus*, aflatoxin, biosynthetic genes, polyketide synthase (PKS) genes

1. Biosynthesis of fungal secondary metabolites requires Cys_6Zn_2 and associated proteins

The proteins required for biosynthesis of many fungal secondary metabolites, including mycotoxins, are encoded by sets of clustered genes (Khaldi *et al.*, 2010). Initial biosynthesis begins with a 'backbone protein', usually a non-ribosomal peptide synthase (NRPS), polyketide synthase (PKS) or dimethylallyl tryptophan transferase (DMAT). Other genes in the cluster encode enzymes that modify the initial biosynthetic product or are involved in its transport from the cell. The ability of fungi to co-ordinately regulate transcription of these clustered genes is known to depend on a single sequence-specific DNA-binding protein of the Cys_6Zn_2 (Gal4 or C6 transcription factor) type unique to the cluster that is able to bind to either a direct or partially palindromic repeat motif in the promoter regions of genes in that gene cluster (MacPherson *et al.*, 2006). Cys_6Zn_2 DNA-binding proteins are involved in transcriptional regulation of many processes in fungi besides secondary metabolite biosynthesis including activation of genes encoding proteins required for both catabolic and anabolic processes (Chang and Todd, 2003).

For regulation of clustered genes the Cys_6Zn_2 DNA-binding proteins must find their correct targets in the promoter sites of the multiple genes in the cluster so that the genes encoding biosynthetic proteins are transcribed in a coordinated fashion. They must also form stable interactions with transcription-associated factors (TAFs) such as the TATA-box binding protein, RNA-polymerase II, and transcription initiation factors. Because most secondary metabolite biosynthesis clusters are inducible, other factors are usually necessary for transcription to proceed normally as has been shown for transcription in yeast (Traven *et al.*, 2006). Previously, gene disruption studies showed that both LaeA and AflR are required for proper transcription of aflatoxin biosynthesis cluster genes (Chang *et al.*, 1995; Kale *et al.*, 2008).

2. LaeA binds to AflR

LaeA, a putative methyltransferase encoded by genes found in most fungi so far sequenced (Yu and Keller, 2005), has been shown to help regulate transcription of many secondary metabolite clusters (Amaike and Keller, 2009; Bayram *et al.*, 2008; Bayram *et al.*, 2009; Bok and Keller, 2004; Bok *et al.*, 2006; Georgianna *et al.*, 2010; Kale *et al.*,

2008; Kim *et al.*, 2009a,b; Kosalkova *et al.*, 2009; Perrin *et al.*, 2007). It has been postulated that LaeA activates secondary metabolic gene clusters by modifying chromatin to bring it from the silent to the active state (Reyes-Dominguez *et al.*, 2010). LaeA's ability to co-ordinately regulate secondary metabolite gene transcription in many different clusters is still not well understood.

Based on yeast two-hybrid assays we determined that AflR, the aflatoxin biosynthesis cluster Cys_6Zn_2 DNA-binding protein, is able to bind to LaeA. In this assay colonies formed on quadruple drop-out medium (highly selective for protein-protein interactions) (Table 1) when yeast were co-transformed with genes encoding LaeA expressed in the Clontech Matchmaker bait plasmid (pGBKT7) and AflR, cloned into the prey plasmid, pGADT7. Colonies were also obtained on quadruple drop-out medium when the N-terminal portion of LaeA was co-expressed with AflR, or LaeA was co-expressed with a region in AflR (aa94 to 300) between its DNA-binding and activation domains (Table 1) (Ehrlich *et al.*, 1998). These results suggest that the binding of LaeA to AflR involves interaction of domains in each protein whose functions had not been previously characterised. Attempts to confirm the interactions by co-immunoprecipitation assays were unsuccessful suggesting that the binding may be too weak to survive the *in vitro* isolation and washing steps involved in the immunoprecipitation assay.

Table 1. Yeast colonies formed on selective media after co-transformation with binding domain and activation domain plasmids expressing LaeA and AflR.[1]

BD	AD	QDO/TDO	DDO
LaeA	AFLA_139360 (AflR)	yes	yes
LaeA	AFLA_128150	yes	yes
LaeAaa1-76	AflR	yes	yes
LaeAaa1-76	control	no	yes
LaeA	control	no	yes
pGBK-laminC	AflR	no	yes
AflRaa94-300	LaeA	yes	yes
AflRaa94-300	control	no	yes

[1] Full-length and partial coding sequences for LaeA and partial coding sequences for AflR were inserted into the binding domain vector pGBKT7 (BD) and AflR or LaeA into the activation domain vector pGADT7 (AD) for yeast two-hybrid analysis by the Matchmaker-2 Kit (Clontech). Selection was on yeast minimal medium plates lacking Leu and Trp (DDO), Leu, Trp, and His (TDO), or Trp, Leu, His, and Ade (QDO). AFLA_128150 was selected from an *Aspergillus parasiticus* cDNA library in pGADT7 using LaeA as the bait. The binding domain control used as insert the coding sequence for laminC while activation domain controls used the pGADT7 vector only.

3. LaeA may bind to AflR-like proteins in other secondary metabolite gene clusters

In a separate experiment using LaeA as bait in the yeast two-hybrid assay to screen an *Aspergillus flavus* cDNA library constructed in pGADT7, we isolated a clone expressing the Cys_6Zn_2-type protein, AFLA_128150, the transcription regulatory factor associated with a secondary metabolite cluster of unknown function [Cluster 52 (Georgianna *et al.*, 2010)] in *A. flavus*. A tBLASTN search of genes in the *A. flavus* genome in GenBank with AFLA_128150 revealed a high scoring hit, AFLA_118300, a Cys_6Zn_2-type protein in another putative LaeA-regulated *A. flavus* secondary metabolite cluster of unknown function, cluster 45 (Table 2). Of the 18 putative gene clusters identified as being regulated by LaeA (Georgianna *et al.*, 2010; N. Fedorova, unpublished results) nine have Cys_6Zn_2-type proteins within the cluster (Table 2). For the other LaeA-regulated clusters it is possible that a Cys_6Zn_2-type protein specific to the cluster activates its genes from a locus outside of the cluster. The aflatrem biosynthesis clusters (Cluster 15 and 32), for example, may be co-regulated by a Cys_6Zn_2-type protein present in Cluster 32. Recently, the dothistromin biosynthesis cluster in *Mycosphaerella pini*, which has a strong resemblance to the sterigmatocystin biosynthesis cluster of *Aspergillus nidulans*, has three loci co-regulated by an ortholog to AflR (Schwelm *et al.*, 2008; Zhang *et al.*, 2007).

We also identified motifs in the promoter regions of many of the genes in these clusters that are of the inverted palindromic nature of Gal4-type protein DNA recognition motifs (Table 2). Like the aflatoxin cluster (Cluster 54), AflR-like binding sites in promoter regions of AFlR-regulated genes with the motif $TCGN_5CGR$ (Ehrlich *et al.*, 1999) are found in some of the promoter regions of genes in some of these clusters (Table 2). The basal palindromic DNA-binding unit recognised by AflR (CGN_xCG) is found in almost all promoter regions of genes in LaeA-regulated clusters with the exception of the genes in cluster 11 (Table 2).

Table 2. Cys_6Zn_2-type transcription factors in putative *Aspergillus flavus* secondary metabolite gene clusters regulated by LaeA.

Cluster[1]	Type (DMAT, PKS or NRPS)	Location	LaeA reg. genes (total)[2]	Promoter AflR sites[3]	Other Gal4 sites[4]	Cys_6Zn_2 factor[5]
11	NRPS	AFLA_022880-023090	8 (12)	1	ggcncgg (3)	AFLA_023040
15	Aflatrem1	AFLA_045490-045540	6 (6)	1	$ccgn_6cgg$ (3)	none
19	DMAT	AFLA_060660-060710	5 (6)	3	none	none
20	PKS	AFLA_062800-062890	6 (10)	0	none	none
21	NRPS	AFLA_064240-064610	21 (25)	17	none	AFLA_064370
23	PKS	AFLA_066820-066980	6 (14)	2	$tcgn_2cga$ (4)	AFLA_066900
24	NRPS	AFLA_069320-069340	2 (3)	0	none	none
31	NRPS	AFLA_094980-095120	13 (15)	4	$tcgn_2cga$ (4)	AFLA_095090
32	Aflatrem2	AFLA_096250-096460	3 (20)	0	$ccgn_6cgg$ (2)	AFLA_096370
35	NRPS	AFLA_101690-101730	4 (5)	2	tcgncga (4)	none
39	PKS	AFLA_108540-108580	5 (5)	1	tcgncga (4)	none
43	PKS	AFLA_116560-116660	9 (11)	4	$tcgn_2cgr$ (4)	none
45	NRPS	AFLA_118300-118460	9 (16)	4	$tcgn_2cgr$ (6)	AFLA_118300
48	NRPS	AFLA_121440-121630	20 (11)	0	$tcgn_6cgr$ (7)	AFLA_121620
50	PKS	AFLA_126660-126730	6 (8)	0	$tcgn_6cgr$ (3)	none
52	PKS	AFLA_128030-128150	6 (12)	5	none	AFLA_128150
54	aflatoxin	AFLA_139140-139410	21 (30)	24	none	AFLA_139360
55	cyclopiazonic acid	AFLA_139460-139500	4 (5)	0	$tcgn_3cgr$ (4)	AFLA_139500

[1] Only putative LaeA-regulated secondary metabolite gene clusters are listed (Georgianna, 2010). Clusters were identified by SMURF (Khaldi, 2010). Cluster type was by homology of backbone genes to those identified in other fungal species. DMAT, dimethylallyl tryptophan synthase; NRPS, non-ribosomal peptide synthase; PKS, polyketide synthase.

[2] Number of genes in the cluster shown to up-regulated by LaeA in microarray assays (N. Fedorova, personal communication) compared to total number of genes in the cluster.

[3] Sites with the motif $tcgn_5cgr$ in the promoter regions.

[4] Gal4 sites were chosen that had a partially palindromic motif. Cys_6Zn_2-type DNA-binding sites are usually short (6 bp) inverted repeats with a 0 to 7 bp spacer region (Reece, 1993). For the analysis, only inverted repeats within 1 kb of the gene's translational start sites were examined.

[5] Protein locus number (GenBank). None indicates that a Cys_6Zn_2 protein was not identified within the cluster boundaries.

4. Some secondary metabolite clusters not regulated by LaeA also contain AflR-like genes

Cys_6Zn_2-type proteins are found in putative secondary metabolite gene clusters in *A. flavus* not thought to be regulated by LaeA (Table 3). Of the 29 SMURF-identified gene clusters (Georgianna *et al.*, 2010) seven contain genes predicted to encode Cys_6Zn_2-type proteins. For four of the LaeA 'un-regulated' clusters, the majority of their genes contain AflR-like binding domains in their promoter regions. It is possible that regulation by LaeA can not be adequately assessed for the genes in these other putative secondary metabolite clusters under the growth conditions used for the microarray studies (Georgianna *et al.*, 2010).

Evidence from chromatin immunoprecipitation (ChIP) studies suggested that in the aflatoxin cluster transcription begins at the polyketide synthase gene promoter and proceeds bidirectionally to the neighbouring promoters of cluster genes (Roze *et al.*, 2007). This suggests that once transcription begins in the gene cluster, RNA polymerase II and its associated transcription activation factors move processively along the chromosome. Such movement would render transcription of gene in clusters a highly efficient process. Another DNA-binding protein, Cre1, a cAMP response element binding protein, was identified as a probable co-regulator of transcription of *nor-1*, the aflatoxin cluster gene for norsolorinic acid reductase (Roze *et al.*, 2004, 2007). Similar involvement of additional DNA-binding transcription factors may be necessary for proper transcriptional regulation of genes in other clusters.

Table 3. Cys_6Zn_2 transcription factors in putative secondary metabolite biosynthesis clusters in *Aspergillus flavus* not shown to be regulated by LaeA.[a]

Cluster	Type	Cluster locus	Cys_6Zn_2 factor	# AflR sites/total
2	DMAT	AFLA_004220-004300	004280	1/9 [b]
4	NRPS	AFLA_005430-005460	none	2/4
5	PKS	AFLA_006170-006190	none	0/2
6	NRPS	AFLA_008700-008810	none	1/7 [b]
7	NRPS, PKS	AFLA_009120-009210	none	2/3[b]
8	NRPS, PKS	AFLA_009980-010060	none	0/8
9	NRPS	AFLA_010550-010640	none	5/10 [b]
13	NRPS	AFLA_038600-038640	none	3/5
17	PKS	AFLA_053760-053870	053760	4/12 [b]
18	NRPS	AFLA_054260-054310	054320	5/6
22	NRPS	AFLA_066690-066740	none	2/6
26	NRPS (2), PKS	AFLA_079320-079430	none	4/10 [b]
28	NRPS	AFLA_082450-082490	none	0/5
29	DMAT	AFLA_084080-084090	none	1/2
30	NRPS	AFLA_090170-090220	none	0/5
33	NRPS	AFLA_096700-096770	none	2/9
34	NRPS	AFLA_100280-100350	100300	3/9
36	PKS (2)	AFLA_104210-104270	104220	1/4 [b]
37	NRPS	AFLA_105170-105190	none	1/6
38	PKS	AFLA_105440-105460	none	0/5
40	PKS	AFLA_112820-112900	112830	1/9
41	PKS	AFLA_114800-114820	none	0/3
42	PKS	AFLA_116160-116260	none	1/11
44	PKS	AFLA_116870-116920	116880	5/10[b]
46	PKS	AFLA_118940-118970	none	0/4
47	NRPS	AFLA_119090-119140	none	3/4[b]
49	PKS	AFLA_125280-125650	125590	2/8
51	PKS	AFLA_127070-127110	none	2/7[b]
53	NRPS	AFLA_135430-135500	none	0/5

[a] Clusters were identified by SMURF and microarray studies (Khaldi *et al*., 2010; N. Fedorova, unpublished results).
[b] AflR site is in the promoter of the backbone gene.

5. AflR or AflR-like proteins may recruit LaeA to the transcription complex

Previous studies revealed that LaeA is not required for *aflR* expression, although *aflR* is expressed in lower amounts in *laeA*-deletion mutants (Bok and Keller, 2004; Bok *et al.*, 2006). Since LaeA is probably not acting as a DNA-binding transcription factor, since it lacks typical DNA-binding domains, its role in regulation of *aflR* transcription is unclear. Introduction of an extra copy of *aflR* into Δ*laeA* mutants at a locus outside of the cluster in *A. nidulans* remediated silencing of ST cluster genes (Bok *et al.*, 2006). Ectopic expression of AflR was also found to overcome nitrate repression of AF cluster genes (Chang *et al.*, 1995). These results suggest that LaeA is not necessary for initiating cluster gene transcription and may be serving simply as a co-activator. We hypothesise that AflR or AflR-like transcription factors may be necessary for recruiting LaeA to the transcription complex. LaeA could then serve as an additional transcription activating factor to overcome repressive factors that prevent *aflR* or *aflR*-like genes from being expressed at wild-type levels. Therefore, our results are consistent with an interaction between LaeA and AflR as being one of the initial steps in activation of genes in the aflatoxin biosynthesis cluster.

Our hypothesis proposes that AflR or an AflR-like Cys_6Zn_2-type protein recruits LaeA to co-regulate the genes in many different secondary metabolite clusters. This hypothesis explains how LaeA is able to only affect the transcription of genes in the Cys_6Zn_2-type protein-regulated cluster and not the transcription of genes adjacent to the cluster. LaeA also was found to bind to another co-activator protein, VeA, a transcription factor that mediates

light-dependent regulation of both sexual development and mycotoxin biosynthesis (Amaike and Keller, 2009; Bayram *et al.*, 2008). Previously, LaeA was postulated to relieve the heterochromatic state of the gene cluster locus (Chiou *et al.*, 2004; Reyes-Dominguez *et al.*, 2010) by recruiting chromatin remodelling proteins and some of the general transcription factors to the pre-initiation site. Our hypothesis is consistent with this model, but further studies are needed to confirm the timing of the interactions of Cys_6Zn_2-type transcription factors and LaeA with chromatin remodelling proteins that specifically activate secondary metabolite gene expression.

References

Amaike, S. and Keller, N.P., 2009. Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*. Eukaryotic Cell 8: 1051-1060.

Bayram, O., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.J., Keller, N.P., Yu, J.H. and Braus, G.H., 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320: 1504-1506.

Bayram, O., Sari, F., Braus, G.H. and Irniger, S., 2009. The protein kinase ImeB is required for light-mediated inhibition of sexual development and for mycotoxin production in *Aspergillus nidulans*. Molecular Microbiology 71: 1278-1295.

Bok, J.W. and Keller, N.P., 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. Eukaryotic Cell 3: 527-535.

Bok, J.W., Noordermeer, D., Kale, S.P. and Keller, N.P., 2006. Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. Molecular Microbiology 61: 1636-1645.

Chang, P.K., Ehrlich, K.C., Yu, J., Bhatnagar, D. and Cleveland, T.E., 1995. Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Applied and Environmental Microbiology 61: 2372-2377.

Chang, P.K. and Todd, R.B., 2003. Metabolic pathway regulation. In: Arora, D.K. (ed.) Handbook of fungal biotechnology. Marcel Dekker Inc., New York, NY, USA, pp. 25-37.

Chiou, C.H., Lee, L.W., Owens, S.A., Whallon, J.H., Klomparens, K.L., Townsend, C.A. and Linz, J.E., 2004. Distribution and sub-cellular localization of the aflatoxin enzyme versicolorin B synthase in time-fractionated colonies of *Aspergillus parasiticus.* Archives of Microbiology 182: 67-79. .

Ehrlich, K.C., Montalbano, B.G., Bhatnagar, D. and Cleveland, T.E., 1998. Alteration of different domains in AFLR affects aflatoxin pathway metabolism in *Aspergillus parasiticus* transformants. Fungal Genetics and Biology 23: 279-287.

Ehrlich, K.C., Montalbano, B.G. and Cary, J.W., 1999. Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. Gene 230: 249-257.

Georgianna, D.R., Fedorova, N.D., Burroughs, J.L., Dolezal, A.L., Bok, J.W., Horowitz-Brown, S., Woloshuk, C.P., Yu, J.J., Keller, N.P. and Payne, G.A., 2010. Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. Molecular Plant Pathology 11: 213-226.

Kale, S.P., Milde, L., Trapp, M.K., Frisvad, J.C., Keller, N.P. and Bok, J.W., 2008. Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. Fungal Genetics and and Biology 45: 1422-1429.

Khaldi, N., Seifuddin, F.T., Turner, G., Haft, D., Nierman, W.C., Wolfe, K.H. and Fedorova, N.D., 2010. SMURF: genomic mapping of fungal secondary metabolite clusters. Fungal Genetics and Biology 47: 736-741.

Kim, H.R., Chae, K.S., Han, K.H. and Han, D.M., 2009a. The *nsdC* gene encoding a putative C2H2-type transcription factor is a key activator of sexual development in *Aspergillus nidulans*. Genetics 182: 771-783.

Kim, H.Y., Han, K.H., Lee, M., Oh, M., Kim, H.S., Xie, Z.X., Han, D.M., Jahng, K.Y., Kim, J.H. and Chae, K.S., 2009b. The *veA* gene is necessary for the negative regulation of the *veA* expression in *Aspergillus nidulans*. Current Genetics 55: 391-397.

Kosalkova, K., Garcia-Estrada, C., Ullan, R.V., Godio, R.P., Feltrer, R., Teijeira, F., Mauriz, E. and Martin, J.F., 2009. The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91: 214-225.

MacPherson, S., Larochelle, M. and Turcotte, B., 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiology and Molecular Biology Reviews 70: 583-604.

Perrin, R.M., Fedorova, N.D., Bok, J.W., Cramer, R.A., Wortman, J.R., Kim, H.S., Nierman, W.C. and Keller, N.P., 2007. Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. PLoS Pathogens 3: e50.

Reece, R.J. and Ptashne, M., 1993. Determinants of binding-site specificity among yeast C6 zinc cluster proteins. Science 261: 909-911.

Reyes-Dominguez, Y., Bok, J.W., Berger, H., Shwab, E.K., Basheer, A., Gallmetzer, A., Scazzocchio, C., Keller, N. and Strauss, J., 2010. Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. Molecular Microbiology 76: 1376-1386.

Roze, L.V., Arthur, A.E., Hong, S.Y., Chanda, A. and Linz, J.E., 2007. The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Molecular Microbiology 66: 713-726.

Roze, L.V., Miller, M.J., Rarick, M., Mahanti, N. and Linz, J.E., 2004. A novel cAMP-response element, CRE1, modulates expression of *nor-1* in *Aspergillus parasiticus*. Journal of Biological Chemistry 279: 27428-27439.

Schwelm, A., Barron, N.J., Zhang, S. and Bradshaw, R.E., 2008. Early expression of aflatoxin-like dothistromin genes in the forest pathogen *Dothistroma septosporum*. Mycological Research 112: 138-146.

Traven, A., Jelicic, B. and Sopta, M., 2006. Yeast Gal4: a transcriptional paradigm revisited. EMBO Reports 7: 496-499.

Yu, J.H. and Keller, N., 2005. Regulation of secondary metabolism in filamentous fungi. Annual Review of Phytopathology 43: 437-458.

Zhang, S., Schwelm, A., Jin, H., Collins, L.J. and Bradshaw, R.E., 2007. A fragmented aflatoxin-like gene cluster in the forest pathogen *Dothistroma septosporum*. Fungal Genetics and Biology 44: 1342-1354.